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Malting quality molecular markers for barley breeding

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Abstract: Barley is one of the world's most important crops. Barley is used for both food and feed and is important for the production of malt. Malt quality is a complex function of barley genetics, environmental conditions during barley growth, and the technological aspects of the malting process. Due to the high heritability of more than half of the malting parameters, barley can be bred for malting quality. Marker-assisted selection (MAS) is a good way to speed up the breeding process. In this study, nine molecular markers were used to screen 115 barley varieties and breeding lines over a four-year period. The results were compared with malting quality parameters. Multicomponent correlation analysis showed a good correlation (R = 0.63; $P \leq 0.01$) between marker screening results and malting quality parameters. In 93 genotypes (80.9%), agreement was found between molecular marker prediction and malting quality determination. Differences between molecular marker screening and malt quality parameters and possible improvements are discussed. The use of molecular markers in MAS is highly appreciated by barley breeders.

Keywords: *Hordeum vulgare* L.; malting quality parameters; marker-assisted selection; polymorphism

Barley (*Hordeum vulgare* L.) is the fourth most widely cultivated cereal species in the world. Barley is used for feed, food, and in the malting industry. In Central Europe, including the Czech Republic, a significant part of harvested barley is used for malt and beer production. Understanding the genetic basis of malt quality is important in malt barley breeding (Fox et al. 2003). Malt quality is of a complex character that is controlled by multiple genes with strong interactions with the environment (Molina-Cano et al. 1997). Recent advances in new technologies have enabled the study of biological processes on different levels. The study of the genome and transcriptome of barley by means of next generation sequencing, DArT and chip analysis sped up the identification of genes and quantitative trait loci (QTLs) involved in malt quality and other features (Duan et al. 2015; Aubert et al. 2018; Kochevenko et al. 2018).

Molecular markers for assessing malting quality traits can provide rapid selection of plants at the early stages of breeding through a study of large populations, thereby increasing the likelihood of detections the desired genetic combinations (Igartua et al. 2000). Several markers have already been developed for example, markers Bmy1 identifying alleles of the gene for β-amylase (Zhang et al. 2007). A marker

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developed by Fechter et al. (2010) allowed to predict enzyme activity and therefore potential malt quality (Fechter et al. 2010). A microsatellite marker EBmac501 showed to be associated with diastatic power, α-amylase and β-amylase activities, and malt extract content (Collins et al. 2003).

In this study, molecular markers developed based on high-throughput Illumina technology were used to screen barley varieties and breeding lines in four-year breeding programme for malting quality. The results of marker screening were compared to the results of malting quality parameters. The novelty is to use new unpublished markers in marker-assisted selection (MAS), providing a good correlation with a targeted level of malt quality.

MATERIAL AND METHODS

Plant material and DNA extraction. In total, 115 barley genotypes were chosen for this study (Table S1 Electronic Supplementary Material (ESM)). Five spring barley varieties were selected as standard: Sebastian, Laudis 550, Petrus, Spitfire (as malting barley), and Aligator (as non-malting barley). Another 23 barley varieties were included and also 87 breeding lines (Table S1 in ESM). Seed samples were collected over four years (2019–2022) and used for molecular marker screening. They were grown on four localities (Stupice, Kroměříž, Žabčice-Obora, Žabčice-Písky), and in those samples malting quality parameters were determined.

For molecular biology analysis, plants were grown in greenhouse conditions. Approximately 30 plants per barley accession were pooled and frozen at –80 °C. Genomic DNA was extracted using cetyltrimethylammonium bromide (CTAB) detergent (Saghai-Maroof et al. 1984). The quality and concentration of DNA were verified using agarose gel electrophoresis. A λ *Hind*III (Fermentas, Lithuania) marker was used as a size and concentration standard.

Marker development and PCR. Based on DNA sequences published by Leišová-Svobodová et al. (2020), twenty-five primer pairs were designed using Primer Express (Ver. 3; Applied Biosystems, USA). After the optimisation and validation process, the final set of nine molecular markers was selected (Table 1). Polymerase chain reaction (PCR) with these primer pairs was carried out in a reaction volume of 15 µL, which consisted of 1× buffer (Qiagen, Germany), 0.33 mM of each dNTP (Invitrogen, Germany), 0.33 µM of each primer (Generi Biotech, Czech Republic), 1 U of *Taq* polymerase (Qiagen, Germany), and 100 ng of DNA template. PCR was performed in a Sensoquest Labcycler (Goettingen, Germany) under the following conditions: an initial denaturing step at 95 °C for 2 min followed by 35 cycles of 30 s at 95 °C, 40 s at primer pair specific temperature (Table 1), then 1 min at 72 °C; and a final extension at 72 °C for 10 min. As a positive control, DNA samples of standard barley varieties, and as a negative control, ultra-pure water were used in each analysis. The separation of the PCR products was carried out using 2% agarose gel electrophoresis and visualized by means of ethidium bromide under UV light. A 50 bp DNA ladder (Fermentas, Lithuania) was used as a size standard.

Micromalting conditions and technological parameter determination. Samples (0.5 kg) of barley grain (fraction over 2.5 mm) were malted in the micro-malting equipment (KVM, Czech Republic). The method used for micro-malting was based on the MEBAK (2011).

Steeping took place in the steeping box for 72 h, with alternating wet stages and air rests. Water and air temperatures were maintained at 14.0 °C. Duration of wet stages and air rests: on the first day, the wet phase took 5 h and the air rest 19 h; on the second day, the wet phase took 4 h and was followed by 20 hour-air rest. On the third day, the water content of the germinating grains was adjusted to 45% by steeping or spraying.

Germination took place in the germination box. The temperature during germination was maintained at 14.0 °C. The total germination time was 72 h.

Kilning took place in a single-floor electrically heated kiln, and the total kilning time was 22 h. The free-drying stage lasted 12 h at 55 °C. During the forced drying stage, the temperature was gradually increased for 6 h up to 75 °C. The curing stage was carried out for 4 h at 80 °C. Malt quality was determined according to the methods described in MEBAK (2011), the EBC Analysis Committee (2010), and American Society of Brewing Chemists (2011). The methods used are shown in Table 2.

Data evaluation. On the basis of the presence or absence of an amplification product, binary data matrices were built. A dissimilarity matrix was computed with DARwin software using the Jaccard coefficient (Perrier et al. 2003; Perrier & Jacquemoud-Collet 2006). A dendrogram was constructed using an unweighted neighbour joining method. A bootstrap analysis with 2 000 replicates was performed to estimate the robustness of a tree.

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Table 1. Molecular markers used in the study

Marker	T_A (°C)	Amplicon length (bp)	Primers	Pos	Ref	Alt	Qual	Variant type	Akref	Akalt	Contig	Length	Bit score	eValue	HitName
DN28011	60	384	DN28011_667F	681	G	A	516.04	stop gained	ak	stop	TRI_DN28011_c0_g1_i1	3 972	2 058.9	0	|XP_003564866.1|structural maintenance of chromosomes protein 2-1 [*Brachypodium distachyon*]
			DN28011_1050R	–											
DN4572_1	60	356	DN4572_1759F	1 779	T	C	2 192.7	synonym SNP	–	–	TRI_DN4572_c0_g1_i1	2 236	1 010.4	0	BAJ90146.1|predicted protein [*Hordeum vulgare* subsp. *vulgare*]
			DN4572_2114R	2 102	G	T	528.36	missence SNP	Arg	Leu					
				2 103	C	T	528.36	synonym SNP	–	–					
DN55074_1	60	421	DN55074_157F	167	G	A	662.98	missence SNP	Arg	Gln	TRI_DN55074_c0_g1_i1	732	942	0	AK358584, predicted protein
				174	A	C	896.72	missence SNP	Val	Ile					
				175	G	A	896.72	synonym SNP	–	–					
			DN55074_577R	–											
DN55074_2	65	363	DN55074_204F	220	C	T	2 438.7	missence SNP	Pro	Ser	TRI_DN55074_c0_g1_i1	732	942	0	AK358584, predicted protein
				225	G	A	2 384.7	synonym SNP	–	–					
			DN55074_566R	552	T	G	1 390.9	missence SNP	Ile	Met					
DN57118	60	444	DN57118_694F	712	G	A	332.12	missence SNP	Ala	Thr	TRI_DN57118_c0_g1_i1	1 967	987.64	0	XP_003564868.1| two-component response regulator ORR26 isoform X1
			DN57118_1137R	1 121	C	T	805.82	missence SNP	Ala	Val					
DN7366	60	318	DN7366_668F	–							TRI_DN7366_c0_g1_i1	202	50.832	8.11E-08	EMS45466.1|Dehydrin DHN3 [*Triticum urartu*]
			DN7366_985R	160	T	A	1 522.9	missence SNP	Ser	Thr					
				161	C	T	1 522.9	missence SNP	Ser	Leu					
DN23879	65	483	DN23879_169F	184	T	C	646.66	missence SNP	Cys	Arg	TRI_DN23879_c0_g1_i1	912	239.97	2.88E-72	XP_003565837.1|proline-rich receptor-like protein kinase PERK9 [*Brachypodium distachyon*]
			DN23879_651R	635	A	G	779.34	missence SNP	Asn	Ser					
DN24964	65	372	DN24964_374F	389	C	T	761.19	missence SNP	Ser	Leu	TRI_DN24964_c0_g1_i1	1 708	658.68	0	KQK04597.1|hypothetical protein BRADI_2g14520v3 [*Brachypodium distachyon*]
				395	G	C	798.91	missence SNP	Trp	Ser					
DN27571_2	65	809	DN27571_162F	1 360	A	T	1 798.3	missence SNP	Thr	Ser	TRI_DN27571_c0_g1_i2	2 499	811.22	0	XP_015636621.1|wall-associated receptor kinase 2 [*Oryza sativa* Japonica group]
			DN27571_970R		TTGT-GTCG	T		frame shift							

T_A – temperature; Pos – position in the contig; Ref – reference; Alt – alternative; Qual – quality; Akref – aminoacid reference; Akalt – aminoacid alternative

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Another approach to studying genetic diversity is based on Bayesian statistics. Software Structure (Ver. 2.3.4) (Pritchard et al. 2000) was used to determine the genetic architecture of barley genotypes. Ten independent runs of 1–13 groups (K = 1 – 13) were performed using 30 000 Markov chain iterations after a burn-in period of 10 000 iterations. The number (K) of clusters into which the sample data (X) were fitted with posterior probability Pr ($X|K$) was estimated using a model with admixture and correlated allele frequency. The optimal value of K was estimated based on ln(P) and on the ΔP calculation, which considers the rate of change in the ln$P$(D) values among successive K runs.

Factor analysis (FA) and canonical correlation analysis (CCA) were performed using Statistica software (StatSoft, Czech Republic). Before analysis, arcsin transformation of the data given in percentages was performed. Factor analysis is a multivariate analysis pointed at an examination of internal contexts and relationships and at a revelation of source data matrix basic structure using latent variables (Meloun et al. 2005). Canonical correlation analysis is used to identify and measure the associations among two sets of variables (Hotelling 1936).

RESULTS

Molecular marker screening. A set of nine molecular markers of malting quality was obtained through the process of optimization and validation. None of the markers is directly connected with a gene involved in malting quality. All are based on single nucleotide polymorphism (SNP) with one exception. One marker introduces a chance of stop codon and another a frameshift. All others represent from one to three synonymous or missense SNPs (Table 1). They were applied to barley varieties and breeding lines within four years. The results are displayed in the Table S1 in ESM.

Cluster analysis based on the data showed three main clusters with bootstrap values of 73%, 2%, 2%, resp. (Figure 1). The genetic structure was evaluated using Bayesian analyses as implemented by STRUC-

Table 2. Factor loadings (Varimax normalized) of evaluated malt quality variables

	Reference	Factor 1	Factor 2	Factor 3	Factor 4
Extract of malt (%)	EBC 2010	0.845	0.231	0.162	0.143
VZ45 (%)	MEBAK 2011	0.798	–0.072	0.051	0.326
Kolbach index (%)	EBC 2010	0.765	0.398	0.060	0.354
Diastatic power (WK u.)	EBC 2010	–0.580	0.043	–0.219	0.567
Apparent final attenuation (%)	EBC 2010	0.115	0.432	–0.714	0.203
Friability (%)	EBC 2010	0.874	0.214	–0.083	–0.089
β-Glucan content of wort (mg/L)	EBC 2010	–0.854	0.006	0.418	–0.017
Viscosity of wort (mPa·s)	EBC 2010	–0.863	–0.025	0.340	–0.007
Haze of wort (90°) (EBC u.)	EBC 2010	–0.086	0.245	0.590	0.082
Protein content in malt (%)	EBC 2010	–0.155	–0.903	–0.215	0.158
Soluble nitrogen in malt (mg/100 g)	EBC 2010	0.677	–0.341	–0.124	0.492
Soluble nitrogen in wort (mg/L)	EBC 2010	–0.848	0.237	–0.066	0.234
Protein content in barley grain (%)	EBC 2010	0.239	–0.828	–0.032	0.237
Starch content in barley grain (%)	EBC 2010	0.184	0.823	0.010	0.149
Sieving fraction over 2.5 mm (%)	EBC 2010	0.114	0.442	0.527	0.213
1 000 grain weight (g)	EBC 2010	0.589	–0.534	0.187	0.147
Falling number (s)	ASBC 2011	–0.135	0.091	–0.122	–0.699
Explained variance		6.201	3.352	1.630	1.584
Proportion to total		0.365	0.197	0.096	0.093

VZ45 – relative extract at 45 °C; numbers red highlighted are statistically significant at the level $P < 0.01$; EBC 2010 – EBC Analysis Commitee (2010); MEBAK 2011 – MEBAK (2011); ASBC – American Society of Brewing Chemists (2011)

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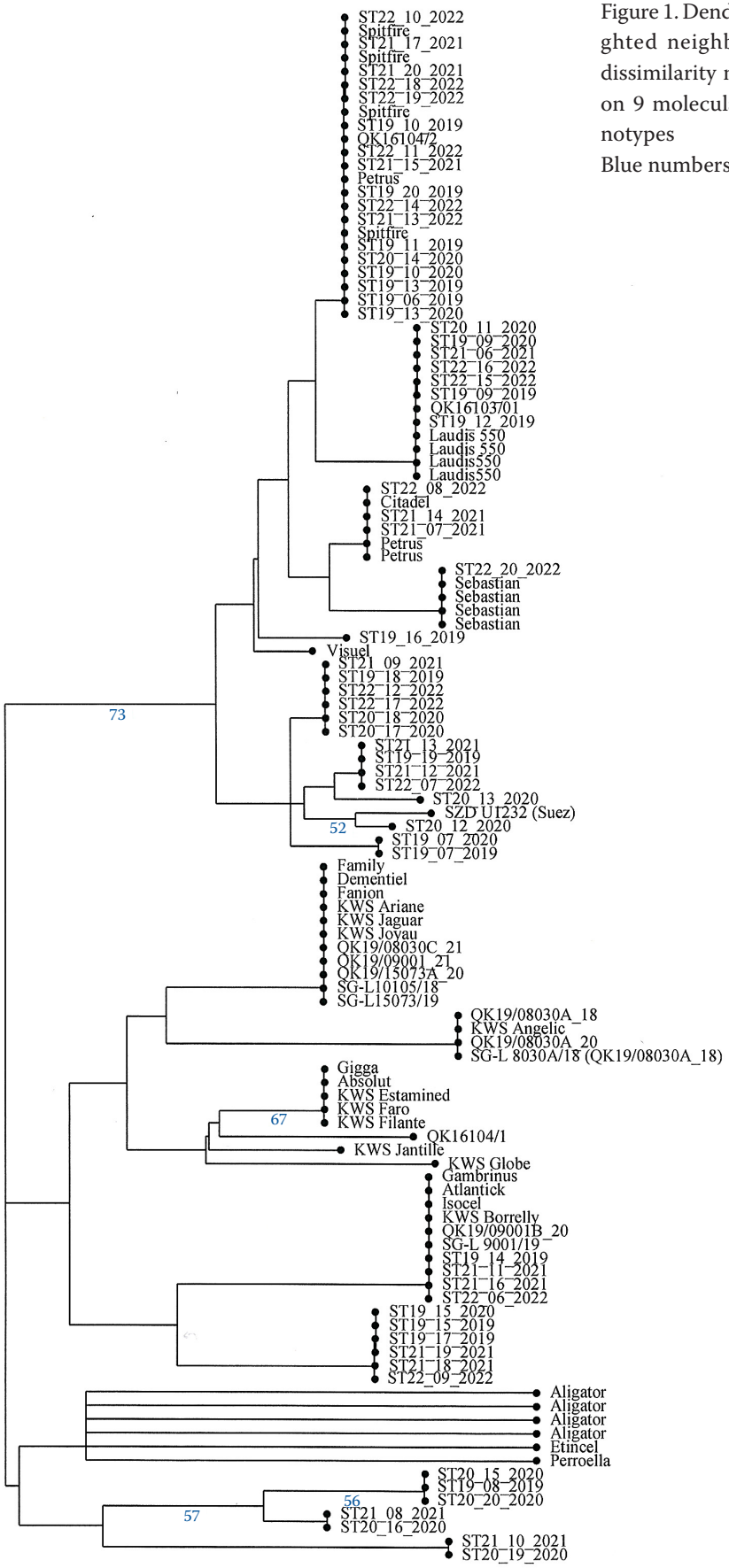

Figure 1. Dendrogram constructed by means of unweighted neighbour joining method applied to the dissimilarity matrix computed using Jaccard indices on 9 molecular marker screening of 115 barley genotypes

Blue numbers – bootstraps values

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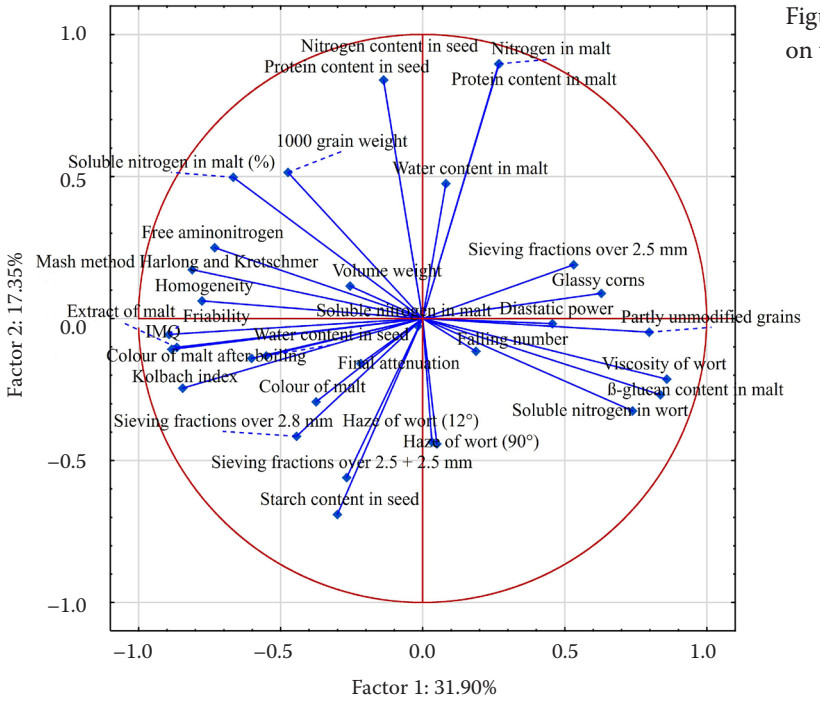

Figure 2. Projection of malt quality variables on the factor plane

TURE software. According to the Δ*P* value, two clusters (K2) were identified among all the analysed samples. One cluster represents barley genotypes with awaited good malting quality (K1), and the second non-malting barley genotypes (K2). The mean α value was 0.03, indicating that most genotypes were not admixed. Nei's heterozygosity index ĥ was 0.19 for K1 and 0.21 for K2, which means that a cluster of non-malting barley genotypes is slightly more variable. The classification of all barley samples into

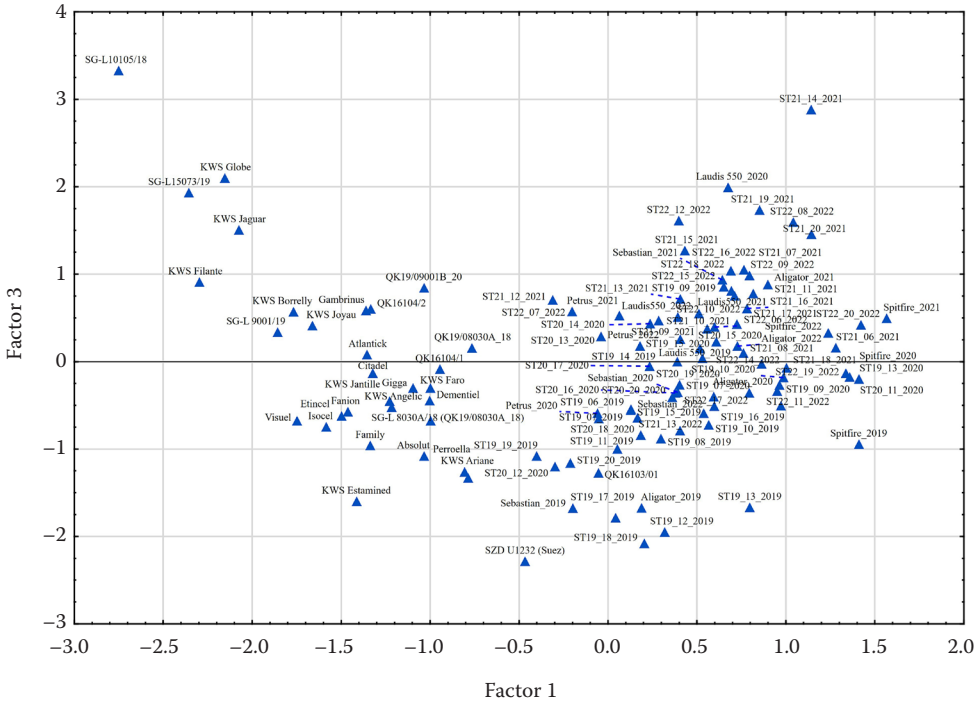

Figure 3. Scatterplot of factor analysis of barley genotypes analysed within the study – Factor 1 against Factor 3

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two *K* clusters was used to help with the interpretation of cluster analysis (Figure 1). The cluster with a high level of bootstrap represented a less variable file of barley genotypes with supposed good malting quality. Another two clusters were formed by non-malting barley genotypes.

In total, 33 malting quality parameters were computed as an average per four localities and four years (Table S2 in the ESM). The first round of factor analysis showed a high redundancy of variables caused by high correlations between pairs or groups of parameters (Figure 2). Consequently, from highly correlated group variables, always the only one was kept into the following analyses. The second round of factor analysis was done with 17 variables. The first three factors represented 66.4% of the total variance. Seven variables contributed significantly to Factor 1: extract of malt, VZ45, Kolbach index, friability, β-glucan in wort, viscosity of wort, and soluble nitrogen in wort (Table 2). All of them represent a quality of wort, a mixture of already lysed polysaccharides and other compounds and a complex of enzymes. Three variables: protein content in malt, protein content in barley grain, and starch content in barley grain contributed significantly to Factor 2. They resume protein and polysaccharide content in barley grain and malt. Factor 3 comprises only one variable. Apparent final attenuation refers to the amount of fermentable polysaccharides and other trace elements and nitrogen compounds, therefore to the wort quality. In Figure 3, there is a scatterplot of cases in an area of Factor 1 and Factor 3. Two clusters could be seen: the left one is formed by genotypes mainly of winter barley usually with the worse level of malt quality, and the dense cluster involves mainly spring malting barley genotypes.

The relationship between molecular marker screening and malting quality parameters was evaluated using canonical correlation analysis. For this analysis, the classification of genotypes into the two *K* clusters was used on the right part and a matrix of 15 malting quality variables without 1 000 grain weight and falling number as they are not directly connected with malting quality on the left part of the equation. The correlation between the two sets of variables was $r = 0.63$ ($P \leq 0.01$). The highest level of canonical weights pertaining to the unique contributions of the respective variables with a canonical variate was identified for VZ45, protein content in malt, and soluble nitrogen in malt (Table 3). The canonical factor loadings representing the overall correlation of the respective variables with the canonical variate showed the highest values for extract of malt, VZ45, friability, β-glucan content in malt, viscosity of wort, and soluble nitrogen in wort (Table 3). Canonical factor scores were used to plot analysed barley genotypes within the plane of correlation variates computed for molecular marker and malting quality data (Figure 4).

Table 3. Canonical correlation analysis – canonical weights and factor structure of evaluated malt quality variables for the significant root 1

Variable	Canonical weight	Factor structure
Extract of malt (%)	–0.084	0.619
VZ45 (%)	0.824	–0.624
Kolbach index (%)	0.176	–0.312
Diastatic power (WK u.)	–0.202	0.300
Apparent final attenuation (%)	0.185	–0.036
Friability (%)	–0.438	–0.746
β-glucan content of wort (mg/L)	0.183	0.722
Viscosity of wort (mPa·s)	–0.044	0.751
Haze of wort (90°) (EBC u.)	0.108	0.126
Protein content in malt (%)	–0.687	–0.130
Soluble nitrogen in malt (mg/100 g)	0.781	–0.435
Soluble nitrogen in wort (mg/L)	0.451	0.845
Protein content in barley grain (%)	0.009	–0.445
Starch content in barley grain (%)	–0.407	0.018
Sieving fraction over 2.5 mm (%)	0.003	–0.089

VZ45 – relative extract at 45 °C

DISCUSSION

For cultivated crops, many molecular markers for genes responsible for important agronomical traits have been developed (Han et al. 1997; Fechter et al. 2010; Gong et al. 2013). Their effectiveness differs in accordance with the genetic makeup of the intended trait. In comparison with monogenic traits, quantitative traits are characterized by low heritability, which leads to a less accurate assessment of their genetic localization and, therefore to the lower effectiveness of developed markers. Moreover, many QTL alleles are difficult to detect when transferred to a different genetic background (Trubacheeva & Pershina 2021). This inaccuracy may be reduced by using several markers per one trait and more different genotypes for sequence treatment and marker design.

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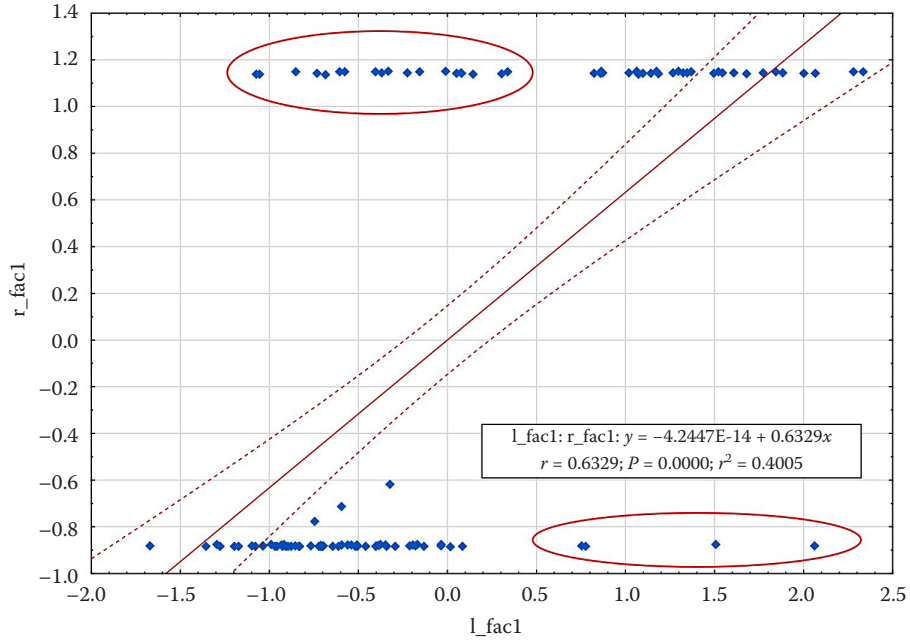

Figure 4. Scatterplot of canonical correlation analysis of barley genotypes analysed within the study – r_fac1 representing molecular marker screening against l_fac1 summarizing malt quality variable results

In this study, we have started with a higher number of proposed molecular markers (25). After validation, nine molecular markers were used to evaluate malting quality in barley varieties and breeding lines. All markers detected polymorphism within examined genotypes, but only in two modes: presence or absence of amplification product. Therefore, two genetic populations were identified: K1 and K2. Factor analysis of malt quality parameter values showed not-so-strict separation of malting and non-malting barley genotypes. Canonical correlation analysis revealed the effectiveness of molecular markers, which was 63.3% ($P < 0.01$).

This value corresponds to a level of correlation ($r = 0.53–0.65$) between several malt quality variables and the cleaved amplified polymorphic sequences (CAPS) marker assay HvTLP8 (Iqbal et al. 2023). Genotypes highlighted with red ellipses in Figure 4 represent the inaccuracy of the developed markers caused by a generally moderate level of malt quality parameters heritability (Han et al. 1997), by technological treatment (Fox et al. 2003), and by the selection of barley genotypes used for marker design study. In this case, the variety Aligator was selected according to the declaration in the list of registered varieties of The Central Institute for Supervising and Testing in Agriculture as a non-malting barley. However, it displayed the level of malt quality of standard malting barley varieties (USJ = 4.7). Therefore, contradictory results between molecular marker screening and malt quality evaluation were found in 22 samples (19%). It means, that the results of screening with the developed molecular markers represent 81% probability of predicting correctly the malt quality in barley breeding lines.

Partial correlations indicated that the markers are mainly associated with three groups of malt parameters: (1) soluble nitrogen in wort, indicating the content of proteins and non-protein compounds getting into wort; (2) friability, β-glucan content in malt, and viscosity of wort representing the level of cytolytic modification; and (3) malt extract and relative extract 45 °C, which represent the levels of protein, starch, and polysaccharides degradation as a result of enzyme activities during malting in particular of α-amylase, β-amylase, glucosidase, and limit dextrinase (Fox et al. 2003). So, the developed markers cover all important malt quality traits. Similar diagnostic ability was found for five kompetitive allele specific PCR (KASP) markers developed on the basis of 9K SNP chip (Genievskaya et al. 2022).

In conclusion, marker-assisted selection proves to be an effective tool in the advancement of malting barley breeding. The developed markers demonstrated 81% probability of reliable prediction of malt quality in breeding lines. Future studies will be aimed at better

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selection of starting barley varieties for another molecular marker design to make marker-assisted selection in malting barley breeding programs more accurate.

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